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# *Corymbiglomus pacificum*, a new glomeromycete from a saline lakeshore in Chile

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Abstract — A new species of the *Glomeromycetes* is diagnosed by bright yellow to dark yellow bi-walled spores (85–131 µm diam.) that form terminally on cylindrical subtending hyphae. The yellow outer spore wall is continuous with the hyphal wall, while the germ tubes emerge during germination from the hyaline inner wall to penetrate the outer wall. Phylogenetic analyses place the new fungus, *Corymbiglomus pacificum*, within the *Diversisporales* next to *C. corymbiforme* and *C. globiferum*. The new species was found in the Araucanía region of southern Chile in the rhizospheric soils of *Ammophila arenaria* at the mouth of Lake Budi, a saline ecosystem where the lake is periodically directly connected to the Pacific Ocean.

Key words — *Glomeromycota*, *Diversisporaceae*, molecular phylogeny, arbuscular mycorrhizal fungi

## Introduction

Budi Lake is an unusual coastal lake in La Araucanía Region of southern Chile. When the lake water levels rise after high winter rainfalls or during exceptionally high tides, the lake connects with the Pacific Ocean, while it is

rarely connected during the summers (Peña-Cortés et al. 2006). During high tides, ocean water enters the lake, giving it a brackish nature (1.5–2.0% salt; Basualto et al. 2006).

*Ammophila arenaria* (L.) Link *(Poaceae)* is one of the dominant pioneer plant species growing on the alluvial-stony lakeshore at the mouth of Budi Lake. This plant, which is distributed worldwide, is often called European beach or European marram grass due to its dominance of many saline dunes in Europe and North Africa. *Ammophila arenaria,* which has high sand burial tolerance, promotes dune formation through sand accumulation. Arbuscular mycorrhizal (AM) fungi are important components for stabilizing such ecosystems through soil aggregation and formation of extensive extraradical mycelial networks. Previous studies have shown that a number of AM fungal species belonging to several genera (e.g., *Acaulospora*, *Diversispora*, *Funneliformis*, *Glomus*, *Racocetra*, and *Scutellospora*) are associated with *A. arenaria* (Błaszkowski 1994; Kowalchuk et al. 2002; Rodríguez-Echeverría & Freitas 2006; Estrada et al. 2011, 2013).

During diversity studies of AM fungi at Budi Lake, an undescribed species was found in the rhizospheric soils of *A. arenaria*. The objective of the present study was to thoroughly investigate the new fungal species through combined morphological and molecular spore analyses and describe the fungus accordingly.

## Materials & methods

### Study site and soil sampling

Lake Budi (approximately 56 $km^2$) is located on the Pacific coastline of the municipality Puerto Saavedra in La Araucanía Region (southern Chile, 38°53′S 73°17′W). Its typical Pacific seaside climate has a narrow temperature range (annual average temperature of 12°C) due to the marine and lacustrine thermoregulation effect; summer maximum and minimum temperatures average 18–20°C and 9–11°C, while winter averages are 13–14°C and 0.5–7°C. The annual average rainfall, concentrated between autumn (March) and early spring (August), is 1350 mm (Peña-Cortés 2008).

Several pioneer species (e.g., *Ammophila arenaria*, *Ambrosia chamissonis* (Less.) Greene, *Polygonum maritimum* L., and *Carpobrotus chilensis* (Molina) N.E. Br.) grow in the lake–ocean transition zone (38°49′34″S 73°23′43″W). Rhizospheric soil was collected from these plant species in 2011 (April, July, October) and 2012 (January). The new fungus was found in *A. arenaria* rhizospheric soils, which were air-dried and stored for AM fungal spore isolation and identification. The soils had a pH ($H_2O$) 8.1, 15 mg $kg^{-1}$ organic carbon, 4.0 mg $kg^{-1}$ available P (as 'Olson'-P), 74.0 mg $kg^{-1}$ available K (as 'ammonium-acetate'-extractable K), and 350.3 mS $m^{-1}$ electrical conductivity.

### AM fungal bait cultures

Although bait cultures were established as described in Oehl et al. (2012), no spores have yet been produced by the new fungus.

**Morphological analyses**

AM fungal spores were separated from the soil samples by a combined wet sieving and specific density/sugar-gradient separation process as described by Sieverding (1991). Spore morphology and subcellular structures were described based on observations of specimens mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG; Koske & Tessier 1983), a mixture of PVLG and Melzer's reagent (Brundrett et al. 1994), a mixture of lactic acid and water at 1:1, Melzer's reagent, and water (Spain 1990). Spore terminology follows that presented for species with glomoid, pacisporoid, diversisporoid or paraglomoid spore formation in Oehl et al. (2005, 2011a) and Mello et al. (2013). Photographs were taken with a digital camera (Leica DFC 290) on a compound microscope (Leitz Laborlux S) using Leica Application Suite Version V 2.5.0 R1 software. Specimens mounted in PVLG and the mixture of PVLG and Melzer's reagent were deposited at the mycological herbaria of ETH Zurich (Z+ZT, Switzerland), of the University of Granada (GDA-GDAC, Spain) and of the Federal University of Pernambuco (URM, Recife, Brazil).

**Molecular and phylogenetic analyses**

After all isolated spores were washed in ultrapure water and sonicated three to four times, crude DNA extracts were obtained from two single spores, extracted from field soil samples taken at the type location in January 2012. The spores were singly placed on a slide in a drop (5–10 µL) of ultrapure water and crushed with a sterile needle. Extracted DNA was used as template for a semi-nested PCR using the primers ITS3+28G2 (White et al. 1990; Silva et al. 2006) and LR1+28G2 (van Tuinen et al. 1998; Silva et al. 2006), consecutively. PCR reactions were carried out in a volume of 50 µL, containing 75 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 200 µM each dNTPs, 1µM of each primer and 2 units of Taq™ DNA polymerase (Fermentas, Maryland, USA); cycling parameters were 5 min at 95 °C (1 cycle), 45s at 94 °C, 1 min at 55 °C, 1 min at 72 °C (40 cycles), and a final elongation of 7 min at 72 °C followed the last cycle. The final amplicons (~690 bp) were purified with the PureLink PCR Purification Kit (Invitrogen™, Carlsbad, USA), sequenced directly or cloned with a CloneJET™ PCR Cloning kit (Fermentas; Carlsbad, USA) following the manufacturer's instructions and sequenced. Sequencing was provided by the Human Genome Research Center (São Paulo, Brazil). Sequence data were compared to public databases (EMBL and GenBank) using BLASTn. The new sequences were deposited in the NCBI database under the accession numbers HG532010–HG532015.

The phylogeny was reconstructed by analyses of the partial LSU rDNA. The AM fungal sequences were aligned in ClustalX (Larkin et al. 2007) and edited with the BioEdit program (Hall 1999) to obtain a final alignment. *Pacispora scintillans* (S.L. Rose & Trappe) Sieverd. & Oehl ex C. Walker et al. was included as outgroup. Prior to phylogenetic analysis, the model of nucleotide substitution was estimated using Topali 2.5 (Milne et al. 2004). Bayesian (two runs over $1 \times 10^6$ generations with a burnin value of 2500) and maximum likelihood (1000 bootstrap) analyses were performed, respectively, in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and PhyML (Guindon & Gascuel 2003), launched from Topali 2.5, using the GTR + G model. Neighbor-joining (established with the model cited above) and maximum parsimony analyses were performed using PAUP*4b10 (Swofford 2003) with 1000 bootstrap replications.

## Results

### Taxonomic analyses

***Corymbiglomus pacificum*** Oehl, J. Medina, P. Cornejo, Sánchez-Castro, G.A. Silva & Palenz., **sp. nov.** Figs 1–8

MycoBank MB 805601

Differs from other *Corymbiglomus* species by the lack of a hyphal mantle.

Type: Chile, La Araucanía Region, Puerto Saavedra (38°49′34″S 73°23′43″W); isolated from the rhizospheric soil of *Ammophila arenaria* at the Budi Lake in close proximity to the Pacific Ocean, 36-3601 (**holotype**, ZT Myc 49005); 36-3602–36-3610 (**isotypes**, ZT Myc 49006); 36-3621 (**isotype**, URM 83531).

Etymology: *pacificum* (Latin), referring to the Pacific Ocean, which is adjacent to the isolation site.

Sporocarps unknown.

Spores formed singly in soil, bright yellow to dark yellow to rarely brownish yellow, globose to subglobose to rarely ellipsoid to irregular, (85–)95–130 (–135) × (75–)85–125(–131) µm diam., with outer and inner walls.

Outer wall smooth, three-layered: outer layer (OWL1) evanescent to semi-persistent, subhyaline to light yellow, 0.6–1.2 µm thick, usually tightly adherent to second layer (OWL2); OWL2 finely laminated, bright yellow to dark yellow to brownish dark yellow, 1.8–3.5(–4.5) µm; inner layer (OWL3) hyaline, thin (0.4–0.7 µm), separable under pressure but usually adherent to OWL2 and then difficult to observe. Outer spore surface partially expands slightly and thus sometimes appears lamellate to slightly pustulate.

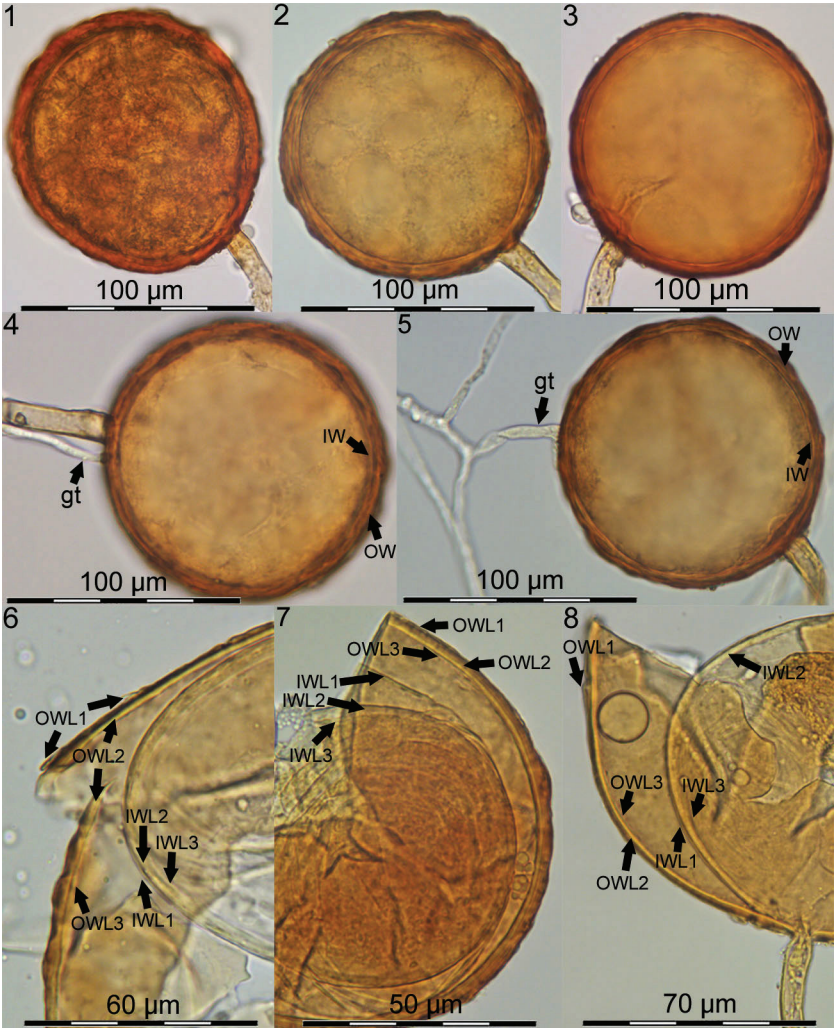
Inner wall hyaline, three-layered: outer layer (IWL1) 0.4–1.0 µm thick (in crushed spores sometimes separating under light pressure from IWL2); central layer (IWL2) 1.5–2.5(–3.0) µm thick; inner layer (IWL3) very thin (0.4–0.7 µm), flexible, showing folds in broken spores, usually adhering to IWL2 and often difficult to observe. IWL2 may stain light yellow to bright dark yellow in Melzer's reagent.

Subtending hypha usually straight (sometimes recurved), 7–12 µm diam. at the spore base, cylindrical (rarely slightly constricted); wall concolorous and continuous with OWL1 and OWL2 and of the same thickness up to (5–)10–25 µm from the spore base, where it tapers to <0.5 µm within 60–120 µm; pore often closed at the spore base by a septal bridge formed by OWL2 and the adherent OWL3, the pore sometimes apparently open, and with the IW that forms de novo during spore formation closing the pore at the spore base.

Germination occurring directly through the outer spore wall after a single germ tube emerges on the outer surface of the inner wall.

Mycorrhiza formation unknown.

Distribution: The new fungal species is restricted thus far to the on the ocean side of Budi Lake in the rhizospheric soil of *Ammophila arenaria.*




Figs 1–8. *Corymbiglomus pacificum*. **1–5**. Uncrushed spores with two walls: outer and inner wall (OW and IW); pigmented subtending hyphae cylindrical to slightly constricted; hyaline germ tubes (gt) penetrating OW; spore surface with rough, lamellate to slightly pustulate appearance due to partial expansion of the outermost wall layer. **6–8**. Crushed spores with triple-layered OW and triple-layered IW (OWL1-3 and IWL1-3); OWL1 evanescent and with lamellate to pustulate appearance; OWL2 laminate (Fig. 7), and IWL2 staining yellow in Melzer's reagent (Fig. 8).

Associated species of *Glomeromycetes* Caval.-Sm. (Oehl et al. 2011c) at the site included *Acaulospora scrobiculata* Trappe, *Glomus macrocarpum* Tul & C. Tul., and *Septoglomus constrictum* (Trappe) Sieverd. et al.

**Molecular analyses**

Phylogenetic analyses of the partial LSU rRNA sequences place *C. pacificum* into the /corymbiforme clade near *C. globiferum* and *C. corymbiforme* and support *Corymbiglomus* with high bootstrap and posterior probabilities values (Fig. 9). The intra-specific variation between LSU rRNA gene sequences for the new species was around 1%. In the BLASTn analysis, the species most closely related to *C. pacificum* was *C. globiferum* with a 96% similarity. Environmental sequences most similar to the new species sequences (97%) were found in roots of *Ixeris repens* (*Asteraceae*) collected from saline coastal beach vegetation in Japan (Yamato et al. 2012).

## Discussion

*Corymbiglomus pacificum* can easily be distinguished morphologically from all other species in the *Glomeromycota* through the combination of spore formation type, spore color, and spore wall structure. The three genera producing bi-walled spores terminally on subtending hyphae are *Pacispora* Sieverd. & Oehl (Oehl & Sieverding 2004), *Corymbiglomus* Błaszk. & Chwat (Błaszkowski 2012, Błaszkowski & Chwat 2013), and some species of *Paraglomus* J.B. Morton & D. Redecker (Morton & Redecker 2001, Oehl et al. 2011b, Mello et al. 2013). However, *Pacispora* species form typically pacisporoid hyphal connections, often with one to multiple hyphal pegs on constricted subtending hyphae, and their inner walls stain purple to dark purple in Melzer's reagent, not found in *Corymbiglomus*. In *Paraglomus*, only one species forms pigmented spores, *P. bolivianum* (Sieverd. & Oehl) Oehl & G.A. Silva (Oehl & Sieverd. 2004, Mello et al. 2013), which have a pitted spore surface.

The three species previously described in *Corymbiglomus* are *C. corymbiforme* (Błaszk.) Błaszk. & Chwat, *C. globiferum* (Koske et C. Walker) Błaszk. & Chwat, and *C. tortuosum* (N.C. Schenck & G.S. Sm.) Błaszk. & Chwat (Błaszkowski 2012, Błaszkowski & Chwat 2013, Błaszkowski 1990, 1995, Koske & Walker 1986, Schenck & Smith 1982). All three are characterized by spores that are individually covered with a hyphal mantle of non-branched or branched hyphae with or without terminal vesiculate swellings (Błaszkowski & Chwat 2013). As the spores of *C. pacificum* are smooth and lack a hyphal mantle, the generic description of *Corymbiglomus* may need emending.

*Corymbiglomus pacificum* (characterized by yellow spores lacking a peridium) is phylogenetically most closely related to *C. globiferum*, which differs morphologically in its orange-brown to 'rich red-brown,' substantially larger spores (150–260 × 150–270 µm, exclusive peridium) (Koske & Walker 1986).

Although *Corymbiglomus* species have not previously been described as having bi-walled spores, it is obvious from Błaszkowski (1995, figs. 6–8) and

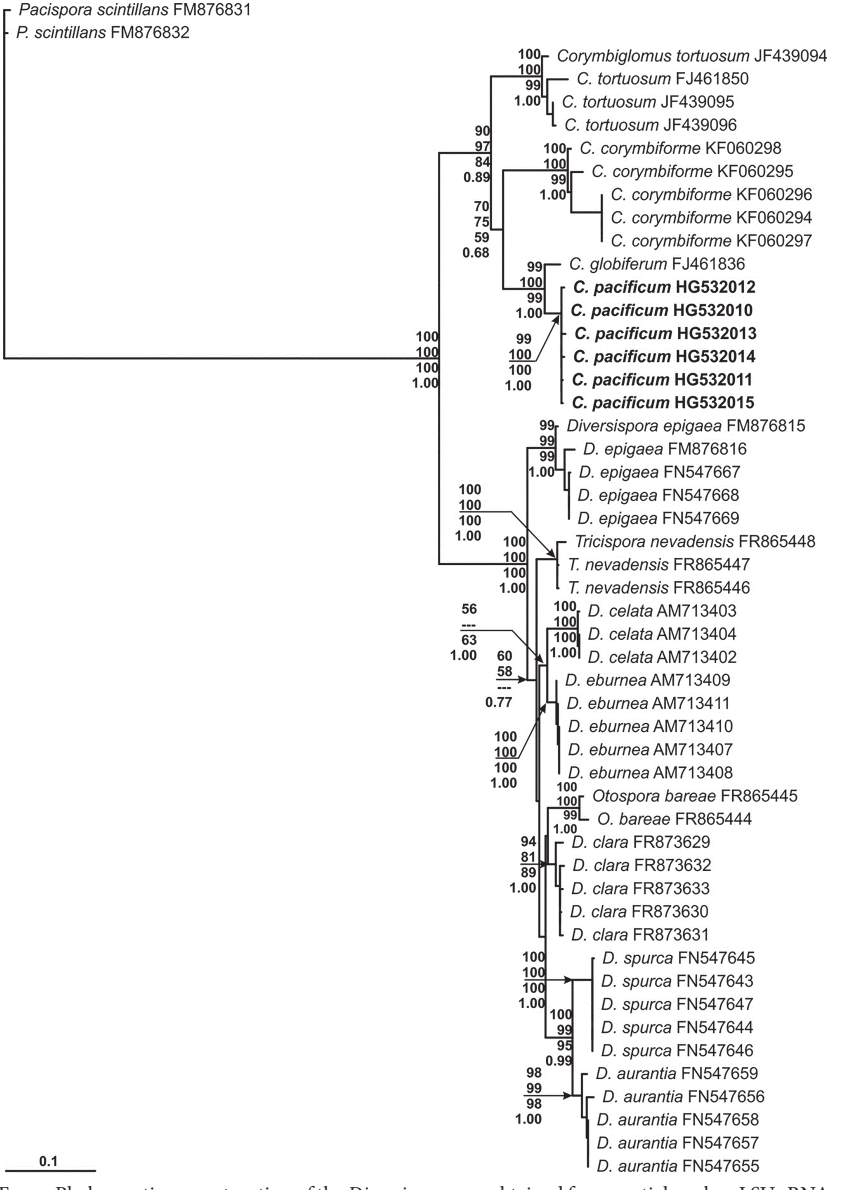



FIG. 9. Phylogenetic reconstruction of the *Diversisporaceae* obtained from partial nuclear LSU rRNA gene sequence analysis. Sequences are labeled with database accession numbers. Support values (from top) are from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses, respectively. Sequences obtained in this work are in bold. Only topologies with ≥50% bootstrap values are shown. (Consistency Index = 0.66; Retention Index = 0.91).

Błaszkowski (2012, fig. 55H) that *C. corymbiforme* has a hyaline inner wall. Also *C. globiferum* was presented with two walls (Koske & Walker 1986, Wu & Sylvia 1993). For *C. tortuosum*, however, more analyses are needed on type and non-type spores, as it appears that spores with one and two walls and similar tortuous spore surfaces have both been identified as *C. tortuosum* (Oehl unpublished), and it is not clear if the type material has also bi-walled spores or belongs in another mono-walled genus.

*Corymbiglomus pacificum,* which is the first *Corymbiglomus* species for which germination has been observed, germinates directly through the spore wall, as in *Pacispora* and *Paraglomus* (Rose & Trappe 1980, Oehl & Sieverding 2004, Oehl et al. 2011a, Mello et al. 2013) and all species in the *Acaulosporaceae* and *Gigasporales*. Lobed structures formed during spore germination (as in *Pacispora*) or persistent germination shields (as in *Acaulosporaceae*, *Dentiscutataceae*, *Intraornatosporaceae*, *Racocetraceae*, and *Scutellosporaceae;* Silva et al. 2008, Oehl et al. 2011d, Mello et al. 2012) have not been observed. Such structures are also not known for *Paraglomus* species. In the *Diversisporaceae,* to which *Corymbiglomus* spp. currently belong, there are two other genera with bi-walled spores, but these form either laterally on or intra-hyphally within the necks of sporiferous saccules (*Otospora* & *Tricispora*), and their germination procedures have not yet been observed. *Diversispora* and *Redeckera* species that form mono-walled spores terminally on hyphae might always germinate through the subtending hyphae, as known for *Diversispora epigaea*, *D. versiformis*, and many mono-walled species of *Glomeraceae* (e.g., http://invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/versiforme, Oehl et al. 2011a).

*Corymbiglomus pacificum* was found at the mouth of Budi Lake (Puerto Saavedra, Chile) close to where the Pacific Ocean periodically floods the lake and lakeshore with seawater that salts the fungal habitat (the rhizospheric soils of *Ammophila arenaria*). Future research will show whether the new fungus is restricted to this particular ecosystem or whether it has a much wider distribution, being more frequently associated with this cosmopolitan sand dune plant species, or is present in other ecosystems without influence of sea water or other salinification. As the four *Corymbiglomus* species have rarely been detected, no conclusion can be drawn about the worldwide occurrence or biogeography of this recently described genus. However, it is remarkable that all four have been reported from sand dunes or other salty ecosystems close to sea or brackish water (e.g., Koske 1987, Koske & Walker 1986, Błaszkowski 1995, Tadych & Błaszkowski 2000). Like *C. pacificum*, *C. corymbiforme* was also first isolated from the rhizosphere of *A. arenaria* (Błaszkowski 1995), while *C. globiferum* was first isolated from American beach grass, the related plant dune species *A. breviligulata* Fernald (Koske & Walker 1986).

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